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Roles of Ca^{2+} in human neutrophil responses to receptor agonists

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Previous studies have concluded that cytosolic Ca^{2+} ($[Ca^{2+}]_i$) transients are essential for neutrophils (PMN) to degranulate and make superoxide anion when challenged with the receptor agonists *N*-formyl-methionyl-leucyl-phenylalanine, platelet-activating factor and leukotriene B_4. This view is based on the profound unresponsiveness of PMN that have their $[Ca^{2+}]_i$ fixed at resting levels by removing storage Ca^{2+} and loading the cells with $\geqslant 20$ μM of a Ca^{2+} chelator, quin2 AM. We too observed this unresponsive state in PMN loaded with 10–32 μM-quin2 AM, fura-2 AM or 1,2-bis-(2-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid (BAPTA). When loaded with $\leqslant 1$ μM fura-2 AM, however, Ca^{2+}-depleted PMN failed to alter $[Ca^{2+}]_i$ appreciably, yet still had substantial degranulation and superoxide-anion-generating responses to the receptor agonists. Function thus did not require $[Ca^{2+}]_i$ transients. Moreover, Ca^{2+}-depleted PMN had 20–35% decreases in receptor numbers for each of the three agonists, and chelator loading of these cells decreased receptor availability by 30–50%. All receptor losses were reversed by incubating PMN with Ca^{2+} at 37 °C, but not at 4 °C, and agonist binding at 4 °C was not influenced by the presence or absence of extracellular Ca^{2+}. Ca^{2+} thus caused PMN to up-regulate their agonist receptors at 37 °C, and the effect persisted at 4 °C regardless of ambient Ca^{2+}. We conclude that Ca^{2+} acts in at least three ways to regulate responses to receptor agonists. First, some pool of (probably cellular) Ca^{2+} maintains receptor expression. Second, $[Ca^{2+}]_i$ transients potentiate, but are not required for, function. The $[Ca^{2+}]_i$ pool may or may not be the same as that influencing receptors. Finally, another pool(s) of Ca^{2+} signals or permits responses. This last pool, rather than $[Ca^{2+}]_i$ transients, appears essential for the bioactions of standard Ca^{2+}-mobilizing stimuli.

INTRODUCTION

Calcium plays a poorly understood role in cellular responses [1,2]. Studies with human polymorphonuclear neutrophils (PMN) exemplify the current situation. PMN contribute to host defences by releasing granule-bound microbiocidal enzymes and superoxide anion (O_2^-). Agents stimulating these responses commonly cause PMN to mobilize storage Ca^{2+}, take up extracellular Ca^{2+}, and thereby increase the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) [3–23]. Moreover, PMN in which $[Ca^{2+}]_i$ is driven to high levels by artificial means (e.g. with Ca^{2+} ionophores, or, in permeabilized cells, high levels of ambient Ca^{2+}) promptly degranulate and make O_2^- [3,12,13,15,24–27]. Finally, $[Ca^{2+}]_i$-blocked PMN do not respond to Ca^{2+}-mobilizing agonists [3–7,9–15,17–24]. On the other hand, some agents activate PMN without altering $[Ca^{2+}]_i$ [3,5,6,8,9,12,20,21,23]; some Ca^{2+}-mobilizing agonists elicit function independently of $[Ca^{2+}]_i$ in PMN primed with other stimuli [3,9,11,24]; and some agents elevate $[Ca^{2+}]_i$, but do not further stimulate PMN [28,29]. The consensus is therefore that PMN have $[Ca^{2+}]_i$-transient-independent as well as $[Ca^{2+}]_i$-transient-dependent response-eliciting pathways, and $[Ca^{2+}]_i$ rises are necessary, although not sufficient, for the degranulating and O_2^--generating actions of standard receptor agonists such as *N*-formylmethionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) [3–12,14–23,25–29]. However, this last conclusion rests on the complete unresponsiveness of $[Ca^{2+}]_i$-blocked PMN, i.e. cells that are suspended in Ca^{2+}-free media, stripped of calcium by Ca^{2+} ionophores and/or millimolar EGTA treatments, and loaded with large amounts of Ca^{2+} chelators. Such PMN may be deprived of other Ca^{2+} pools needed for assembly of complex responses. We show here that PMN depleted of Ca^{2+} by gentler methods (e.g. incubation with 1 μM-EGTA) and loaded with small amounts of a different intracellular chelator, fura-2 AM, are unable to alter $[Ca^{2+}]_i$ appreciably, yet mount prominent exocytotic and oxidative-metabolism responses to fMLP, PAF and LTB_4. Moreover, these PMN had decreased numbers of receptors for each of the three agonists. Ca^{2+} thus regulates receptor availability in PMN, and this effect may contribute to depressing cell responses. In any event, Ca^{2+} acts at various sites in the PMN response mechanism, and the unresponsiveness of $[Ca^{2+}]_i$-blocked PMN cannot be ascribed simply to the absence of $[Ca^{2+}]_i$ transients.

EXPERIMENTAL

Materials

We prepared [^{3}H]PAF (56 Ci/mol) and LTB_4 [30,31], and purchased the following: [^{3}H]LTB_4 (180 Ci/mmol) and [^{3}H]-fMLP (53.6 Ci/mmol) (DuPont–New England Nuclear, Boston, MA, U.S.A.); PAF (Bachem Biosciences, Philadelphia, PA, U.S.A.); fMLP (Peninsula Laboratories, San Carlos, CA, U.S.A.); fura-2 AM, quin2 AM, 1,2-bis-(2-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid (BAPTA) and fura-2 and quin2 pentapotassium salts (Molecular Probes, Junction City, OR, U.S.A.); fatty acid-free type II BSA, cytochalasin B (CB) and type IV cytochrome *c* (Sigma Chemical Co., St. Louis, MO, U.S.A.); superoxide dismutase (SOD) (Diagnostic Data, Mountain View, CA, U.S.A.); silicone oil (General Electric, Waterford, NY, U.S.A.); and Ready Safe scintillation fluid (Beckman Instruments, Fullerton, CA, U.S.A.). Our modified Hanks' buffer contained 154 mM-NaCl, 0.75 mM-Na_2HPO_4, 5.4 mM-KCl,

Abbreviations used: $[Ca^{2+}]_i$, cytosol free Ca^{2+} concentration; PMN, polymorphonuclear neutrophilic leucocytes; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); LTB_4, leukotriene B_4; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; CB, cytochalasin B; O_2^-, superoxide anion; SOD, superoxide dismutase.

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0.74 mM-KH_2PO_4, 25 mM-Tris and 1 μM-EGTA (pH 7.4). $CaCl_2$ was added to this buffer where indicated. Agonists and ^{3}H-labelled ligands were taken up in Hanks' buffer supplemented with BSA (2.5 mg/ml) and added to PMN suspensions so that the final BSA concentration was 125 μg/ml.

Methods

Normal human donor blood was drawn into syringes containing EDTA (10 mM) plus heparin (10 units/ml), diluted with equal volumes of Ca^{2+}-free normal saline, and centrifuged over Ficoll/Hypaque discontinuous gradients [30]. The isolated granulocyte layer was twice exposed to hypo-osmotic medium (24 ml of 0.1 % NaCl; 25 s each; 4 °C) and washed in Ca^{2+}-free normal saline to obtain a final preparation of > 95 % PMN, < 5 platelets per 100 PMN, and no erythrocytes. Then 5×10^7 PMN were incubated in our standard Ca^{2+}-free EGTA (1 μM)-containing Hanks' buffer at 37 °C for 30 min. Where indicated, fura-2 AM, quin2 AM or BAPTA was added at the start of this incubation. PMN were then washed twice in this buffer (4 °C) and processed further as needed in each individual assay. As determined with fura-2 (pentapotassium salt), our (1 μM-EGTA-containing) Hanks' solution had < 17 nM-Ca^{2+}.

Bioassays

For degranulation, 1.3×10^6 PMN in 0.5 ml of Hanks' buffer were incubated with 0 or 1.4 mM-Ca^{2+} for 16–18 min (37 °C), treated with 2.5 μg of CB for 2–4 min, challenged for 5 min, placed on ice, and centrifuged (200 *g*; 5 min; 4 °C). Isolated supernatants were assayed for lysozyme, β-glucuronidase and lactate dehydrogenase [30]. Results are reported as net enzyme release, i.e. the percentage of total cellular enzyme released by stimulated PMN minus that released by BSA-treated PMN. None of the stimuli or chelators caused net release of the cytosol marker, lactate dehydrogenase. Net lysozyme and β-glucuronidase release therefore represented degranulation rather than cell lysis.

For O_2^-, 10^7 PMN in 1 ml of buffer were incubated with 0 or 1.4 mM-Ca^{2+} for 16–18 min (37 °C), treated with 5 μg of CB for 2–4 min, and challenged for 10 min in the presence of 50 nmol of cytochrome *c* with or without 50 μg of SOD. Differences in A_{550} between stimulated PMN suspensions containing 0 or 50 μg of SOD were monitored with a split-beam U 2000 Hitachi spectrophotometer. Results are given as the maximal rate (nmol/min) of SOD-inhibitable O_2^- formation after challenge, assuming a molar absorption coefficient of 21000 for the difference between oxidized and reduced cytochrome *c*.

For Ca^{2+} transients, fura-2 AM-preloaded PMN (10^7) in 1 ml of buffer were incubated with 0 or 1.4 mM-Ca^{2+} for 16–18 min (37 °C), treated with 5 μg of CB for 2–4 min, and challenged for 2.5 min. Suspensions were alternatively excited at 340 and 380 nm and monitored at 510 nm [30]. $[Ca^{2+}]_i$ was estimated by comparisons with standard Ca^{2+}/EGTA/fura-2 (pentapotassium salt) solutions. To determine cell content of fura-2 and quin2, chelator-loaded PMN suspensions were treated with 0.1 % Triton X-100 plus 5 mM-Ca^{2+} and assayed for intensity of fluorescence (fura-2: 340 nm excitation, 510 nm emission; quin2: 339 nm excitation, 492 nm emission). Results were compared with standard solutions of fura-2 and quin2 pentapotassium salts in Hanks' buffer (5 mM-Ca^{2+}).

Ligand binding

For this, 10^7 PMN in 1 ml of buffer were incubated with 0 or 1.4 mM-Ca^{2+} for 20 min, diluted with 8 vol. of 4 °C buffer (Ca^{2+} concn. as in the original incubation), centrifuged (400 *g*; 4 min; 4 °C); and resuspended in 4 °C buffer (Ca^{2+} as in the original incubation). All binding was done at 4 °C. Either 2.5×10^6 (for LTB_4) or 5×10^6 PMN (for PAF or fMLP) were incubated for 40 min (for LTB_4) or 60 min (for PAF or FMLP) in 1 ml of buffer containing 10 pM-[^{3}H]LTB_4 with or without 100 nM-LTB_4, 10 pM-[^{3}H]PAF with or without 100 nM-PAF, or 63.2 pM-[^{3}H]-fMLP with or without 6.32 μM-fMLP. Suspensions were centrifuged (12000 *g*; 1 min; 4 °C) through 400 μl of silicone oil and separated into pelleted material and supernatant fluids. Pellets and 200 μl samples of supernates were incubated with 0.5 ml of methanol for 10 min, mixed with 2 ml of Ready Safe scintillant, and counted for ^{3}H radioactivity. LTB_4 and PAF specific binding under these conditions had reached equilibrium, and was fully reversible and readily saturated [30,31]. We found that the binding of 63.2 pM-[^{3}H]fMLP reached apparent equilibrium within 60 min. Equilibrium binding was proportional to PMN cell counts between 10^6 and 4×10^7 cells/ml, reversed by > 95 % within 90 min of adding 6.32 μM-fMLP, and saturated with increasing amounts of fMLP. Scatchard analyses used the LIGAND program and data from PMN incubated with $\frac{1}{2}$-log incrementally increasing concentrations of [^{3}H]fMLP + fMLP (63.2 pM–6.32 μM), [^{3}H]PAF + PAF (10 pM–100 nM plus 200 nM), and [^{3}H]LTB_4 + LTB_4 (10 pM–1 μM).

RESULTS

Ca^{2+} transients

PMN were loaded with 1 μM-fura-2 AM in our Ca^{2+}-free medium ([EGTA] = 1 μM), washed twice and resuspended in this

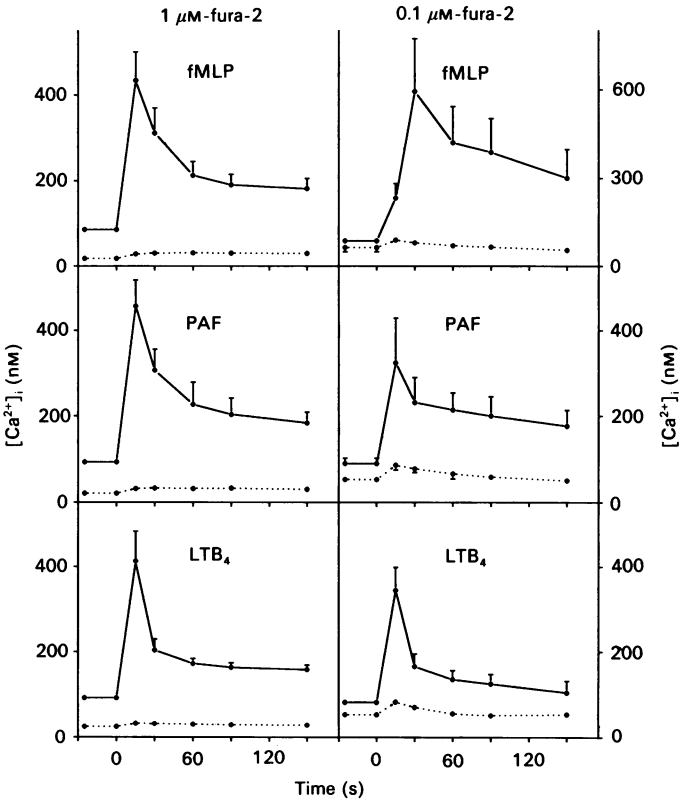

Fig. 1. $[Ca^{2+}]_i$ in agonist-stimulated PMN

Cells were loaded with 1 μM (left panels) or 0.1 μM (right panels) fura-2 AM in Ca^{2+}-free buffer, washed twice and resuspended (10^7 cells/ml) in this buffer, incubated with 0 (broken lines) or 1.4 mM-(continuous lines) Ca^{2+} for 16–18 min, treated with 5 μg of CB/ml for 2–4 min, and challenged with 1 μM-fMLP, -PAF or -LTB_4. Note the different ordinate scale for the upper right panel. Data are means (± S.E.M. where included) for 5–7 experiments.

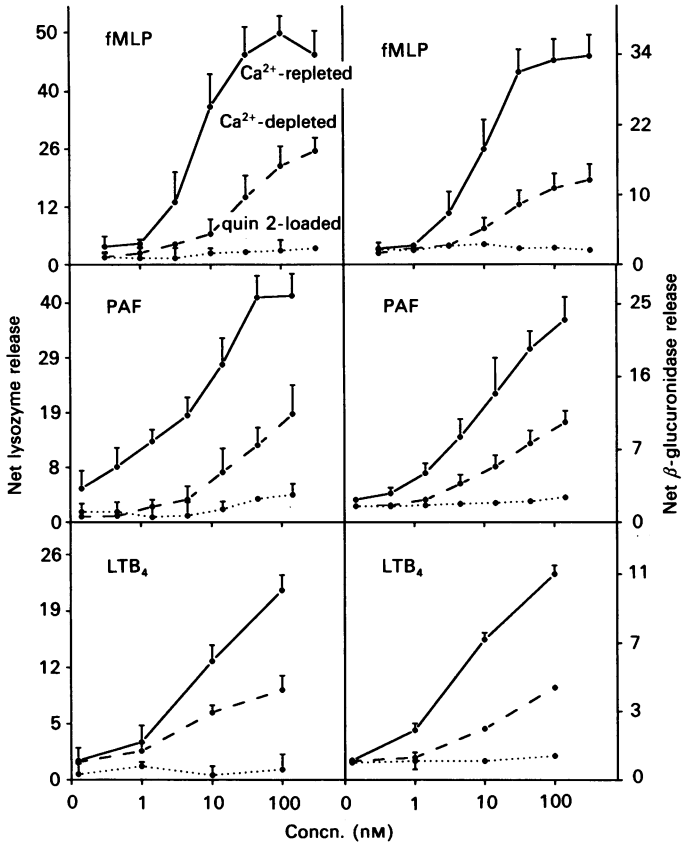

Fig. 2. Effects on Ca^{2+} of PMN degranulation (lysozyme release, left panels; β-glucuronidase release, right panels) responses

Cells were loaded with 0 or 32 μM-quin2 AM (final concn. 2 nmol/10^7 PMN) in Ca^{2+}-free buffer, washed twice and resuspended (2.5×10^6 cells/ml) in this buffer, incubated with 0 (Ca^{2+}-depleted or quin2-loaded) or 1.4 mM- (Ca^{2+}-repleted) Ca^{2+} for 16–18 min, treated with 5 μg of CB/ml for 2–4 min, and challenged with the indicated stimuli. The responses of quin2-loaded PMN were fully restored to levels of Ca^{2+}-repleted PMN by a 20 min incubation with 1.4 mM-Ca^{2+} (results not shown). Note the different ordinate scale for each panel. Data are means (±S.E.M. where included) for 7–9 experiments.

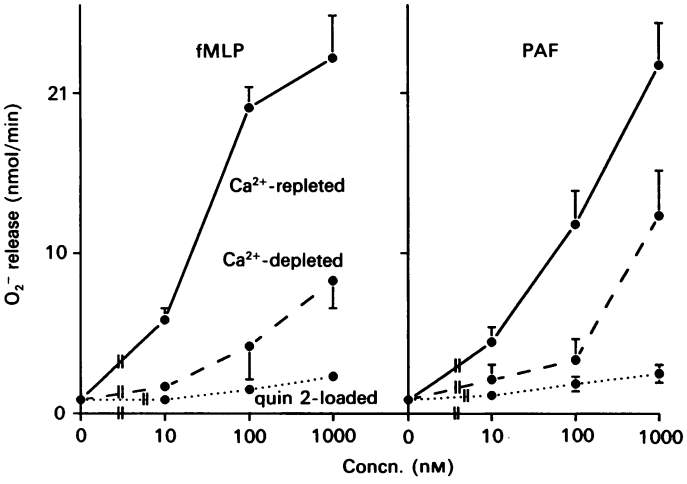

Fig. 3. Effects of Ca^{2+} on PMN O_2^--generation responses

Ca^{2+}-repleted, Ca^{2+}-depleted and quin2-loaded PMN were prepared as described in Fig. 2 legend. Cells were incubated with 5 μg of CB/ml for 2–4 min and challenged with the indicated stimuli. The responses of quin2-loaded Ca^{2+}-depleted PMN were fully restored to the levels of Ca^{2+}-repleted PMN by a 20 min incubation with 1.4 mM-Ca^{2+} (results not shown). Data are means (±S.E.M. where included) for 7–10 experiments.

Table 1. Effects of Ca^{2+} on O_2^- release by stimulated PMN

PMN were loaded with 32 μM-quin2 AM, 32 μM-fura-2 AM or 10 μM-BAPTA, washed twice, incubated with 0 or 1.4 mM-Ca^{2+} for 16–18 min, treated with CB for 2–4 min, and challenged with 1 μM-fMLP, 1 μM-PAF or 100 nM-LTB_4. Results are given as the maximal rate (nmol/min per 10^7 PMN) of O_2^- release, as means±S.E.M. for ≥ 4 experiments.

	Chelator			
Stimulus, Ca^{2+}	None	Quin2	Fura-2	BAPTA
fMLP, $+Ca^{2+}$	22.4±4.8	22.4±6.8	18.6±3.7	26.6±2.1
fMLP, $-Ca^{2+}$	7.7±1.9	0	0.3±0.2	0
PAF, $+Ca^{2+}$	22.7±7.3	15.3±5.7	14.4±6.6	24.6±2.9
PAF, $-Ca^{2+}$	12.6±3.1	0	0	0
LTB_4, $+Ca^{2+}$	7.0±2.2	6.1±0.9	7.4±2.1	6.8±1.3
LTB_4, $-Ca^{2+}$	1.8±0.9	0	0	0

Table 2. Effects of Ca^{2+} on lysozyme release by stimulated PMN

PMN were loaded with 32 μM-quin2 AM, 32 μM-fura-2 AM or 10 μM-BAPTA, washed twice, incubated with 0 or 1.4 mM-Ca^{2+} for 16–18 min, treated with CB for 2–4 min, and challenged with 316 nM-fMLP, 200 nM-PAF or 100 nM-LTB_4. Results are given as net lysozyme release (%), as means±S.E.M. for 3–8 experiments. Net β-glucuronidase release was similarly effected by Ca^{2+} and the chelators.

	Chelator			
Stimulus, Ca^{2+}	None	Quin2	Fura-2	BAPTA
fMLP, $+Ca^{2+}$	49.8±8.1	55.6±8.1	64.3±6.7	65.8±4.8
fMLP, $-Ca^{2+}$	25.5±3.2	0.0±0.8	9.2±3.4	4.0±2.3
PAF, $+Ca^{2+}$	43.9±4.3	41.0±9.6	43.2±6.3	46.5±4.8
PAF, $-Ca^{2+}$	15.7±5.5	3.3±2.1	4.9±1.9	2.6±2.2
LTB_4, $+Ca^{2+}$	21.6±1.9	17.2±2.0	32.2±2.6	24.4±2.1
LTB_4, $-Ca^{2+}$	9.2±1.8	−0.7±1.9	1.6±1.1	2.4±1.1

medium, and incubated for 20 min at 37 °C. After this, 10^7 cells contained 700 pmol of fura-2 and had a baseline $[Ca^{2+}]_i$ of 23±5 nM (mean±S.E.M.). $[Ca^{2+}]_i$ rose minimally (to < 35 nM) in response to 100 pM–1 μM of fMLP, PAF or LTB_4 (Fig. 1, left-hand panels, broken lines). Hence these PMN were Ca^{2+}-depleted. Contrastingly, the same PMN, when incubated for 20 min with 1.4 mM-Ca^{2+} (37 °C), were Ca^{2+}-repleted: their resting $[Ca^{2+}]_i$ was 69±15 nM, and this rose to > 425 nM within 15 s of challenge with 1 μM of the receptor agonists (Fig. 1, left-hand panels, continuous lines). Ca^{2+}-repleted PMN showed essentially normal dose–response curves over an agonist range of 0.3 nM–1 μM [32]. The same general results occurred in PMN loaded with 0.1 μM-fura-2 AM (80 pmol/10^7 PMN), except that: (*a*) fura-2 fluorescence was difficult to detect and more variable; (*b*) $[Ca^{2+}]_i$ rose to ~ 40–50 nM following stimulation of Ca^{2+}-depleted PMN with 1 μM of the agonists; and (*c*) the responses of Ca^{2+}-repleted PMN developed somewhat more slowly and were more sustained (Fig. 1, right-hand panels). Evidently, then, fura-2 tends to buffer $[Ca^{2+}]_i$ changes and thereby may help to fix $[Ca^{2+}]_i$ at or near to baseline values (see [10]). In any case, Ca^{2+}-depleted PMN loaded with ≥ 0.1 μM-fura-2 AM exhibit little or no change in $[Ca^{2+}]_i$ after fMLP, PAF or LTB_4 challenge.

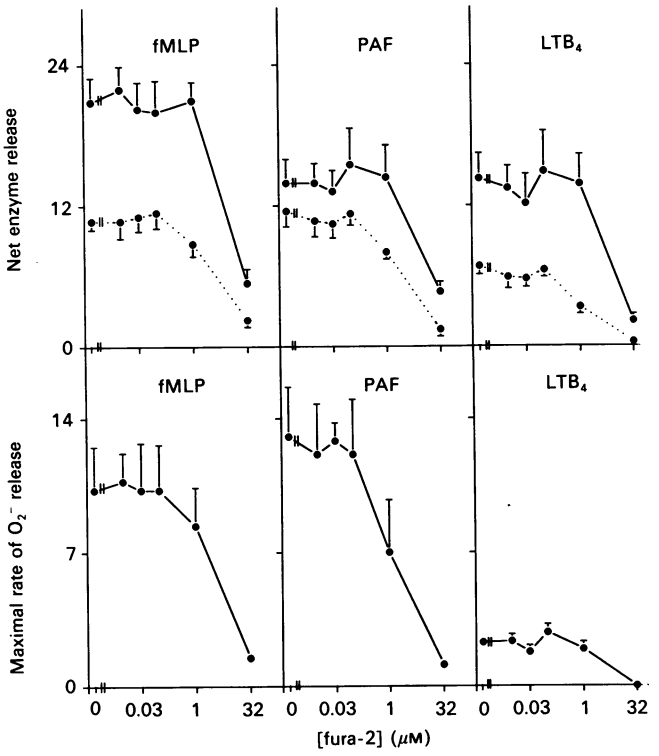

Fig. 4. Effects of fura-2 on PMN degradation (lysozyme release, continuous lines in upper panels; β-glucuronidase release, broken lines) and O_2^- generation (lower panels) responses

PMN were loaded with 0–32 μM-fura-2 AM in Ca^{2+}-free buffer, washed twice and resuspended (2.6×10^6 cells/ml for degranulation; 10^7 cells/ml for O_2^- generation) in this buffer, and challenged with 1 μM-fMLP, -PAF or -LTB_4. All responses were restored to the levels of Ca^{2+}-repleted PMN by a 20 min incubation with 1.4 mM-Ca^{2+} (results not shown). Data are means ± S.E.M. for 5–11 experiments.

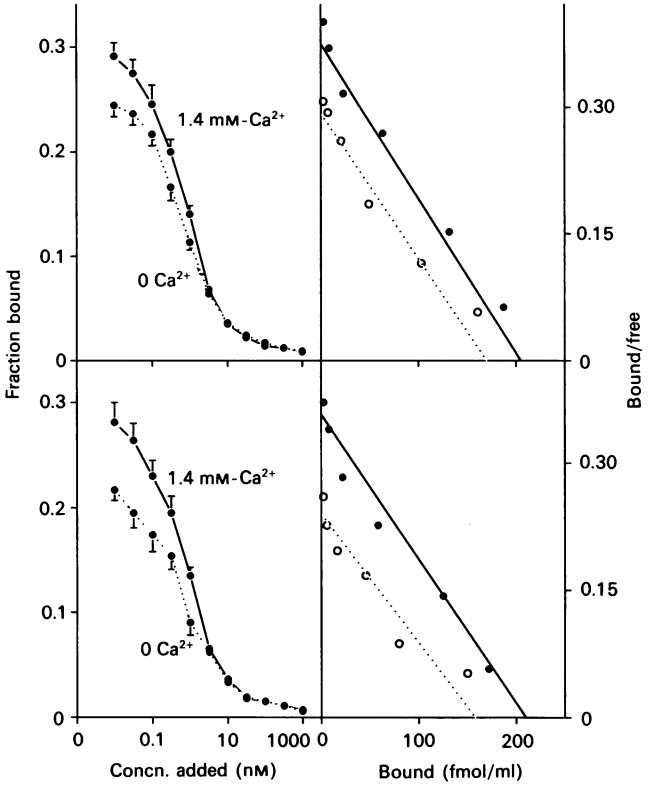

Fig. 5. Effects of Ca^{2+} on PMN binding of LTB_4

Cells were loaded with 0 (upper panels) or 10 μM- (lower panels) BAPTA in Ca^{2+}-free buffer, resuspended (5×10^6 cells/ml) in this same buffer, incubated at 37 °C with 1.4 mM- (continuous lines) or 0 (broken lines) Ca^{2+} for 20 min, centrifuged (400 *g*; 4 min; 4 °C), resuspended in 4 °C buffer containing 1.4 mM- or 0 Ca^{2+} as in the original incubation, and assayed for [^{3}H]LTB_4 binding (4 °C). Panels on the left give displacement curves for the binding of 10 pM–1 μM-[^{3}H]LTB_4 plus LTB_4 (means ± S.E.M.). Panels on the right give Scatchard plots for high-affinity binding and use data corrected for non-specific and low-affinity binding with the LIGAND program. Low-affinity binding parameters did not vary significantly between the treatment groups. Data are means for 11 experiments.

Table 3. Effects of Ca^{2+} on the specific binding of receptor agonists to PMN

PMN were loaded (37 °C) with no chelator, 32 μM-quin2 AM, 32 μM-fura-2 AM or 10 μM-BAPTA for 30 min, washed twice (4 °C), incubated (37 °C) with 0 or 1.4 mM-Ca^{2+} for 20 min, resuspended in 4 °C buffer containing Ca^{2+} as in the original incubation, and assayed (4 °C) for the specific binding of the indicated agonist. Results are given as the percentage of recovered radiolabel specifically bound per 5×10^6 PMN (means ± S.E.M.; $n \geq 10$): * indicates $P < 0.05$ compared with the corresponding values for PMN incubated with no chelator and 1.4 mM-Ca^{2+}; ** indicates $P < 0.05$ compared with the corresponding values for PMN incubated with no chelator and 0 mM-Ca^{2+}. Statistical significance of differences was analysed by Student's paired *t* test.

	Agonist		
Chelator, Ca^{2+}	fMLP	PAF	LTB_4
None, $+Ca^{2+}$	0.55 ± 0.05	4.8 ± 0.5	25.0 ± 1.4
None, $-Ca^{2+}$	0.41 ± 0.03*	3.6 ± 0.7*	20.2 ± 1.1*
Quin2, $+Ca^{2+}$	0.71 ± 0.18	5.1 ± 0.7	26.5 ± 1.1
Quin2, $-Ca^{2+}$	0.40 ± 0.04*	3.0 ± 0.4*	17.1 ± 1.5**
Fura-2, $+Ca^{2+}$	0.62 ± 0.06	4.4 ± 0.6	26.3 ± 4.8
Fura-2, $-Ca^{2+}$	0.38 ± 0.02*	3.2 ± 0.7*	17.5 ± 2.8**
BAPTA, $+Ca^{2+}$	0.48 ± 0.06	4.9 ± 0.4	23.3 ± 1.6
BAPTA, $-Ca^{2+}$	0.28 ± 0.04**	2.8 ± 0.6**	17.6 ± 1.1**

Functional responses

Relative to Ca^{2+}-repleted PMN, Ca^{2+}-depleted PMN had 30–75% decreases in agonist-induced degranulation and O_2^--generation responses. After loading with 32 μM-fura-2 AM, 32 μM-quin2 AM or 10 μM-BAPTA, Ca^{2+}-depleted PMN were virtually unresponsive to fMLP, PAF and LTB_4 (Figs. 2 and 3). Ca^{2+}-repletion of the latter cells reversed all inhibition (Tables 1 and 2). These results agree with studies showing that Ca^{2+}-depleted chelator-loaded PMN are unresponsive to receptor agonists [3–24]. In further study of this inhibition, however, we found that Ca^{2+}-depleted PMN loaded with ≤ 1 μM-fura-2 AM had little loss in responsiveness beyond that achieved with Ca^{2+}-depletion alone (Fig. 4). In particular, Ca^{2+}-depleted PMN loaded with 0.1–1 μM-fura-2 AM did not raise $[Ca^{2+}]_i$ appreciably (Fig. 1), yet released substantial amounts of lysozyme, β-glucuronidase and O_2^- after treatment with 1 μM (Fig. 4) or 10–100 nM (results not shown) of fMLP, PAF and LTB_4. The profoundly unresponsive state produced by high concentrations of intracellular Ca^{2+} chelators thus cannot be fully explained by the blockage of Ca^{2+} transients.

Agonist binding

Ca^{2+}-depleted PMN had 20–35% decreases in their specific binding capacities for [^{3}H]fMLP, [^{3}H]PAF and [^{3}H]LTB_4; chel-

Table 4. Effects of Ca^{2+} on PAF and LTB_4 high-affinity binding to PMN

PMN were Ca^{2+}-depleted and chelator-loaded, washed twice, incubated with 0 or 1.4 mM-Ca^{2+} at 37 °C for 20 min, resuspended in the same buffer at 4 °C, and incubated (4 °C) with various concentrations of the indicated agonist. Results are given as receptor numbers per cell (R_t) and association constants (K_a) (±S.E.M.; $n \geq 10$), as determined by the LIGAND program. LIGAND indicated that each agonist bound to high- and low-affinity sites under all conditions ($P < 0.05$, F-distribution). Results are for high-affinity binding sites only. Low-affinity binding parameters showed no significant differences between the treatment groups.

	$10^{-4} \times R_t$ (receptors/PMN)		$10^{-9} \times K_a$ (M)	
Agonist, chelator	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
PAF, quin2	0.23±0.05	0.13±0.03	2.5±0.8	3.3±1.6
LTB_4, none	4.9±0.4	4.0±0.4	1.8±0.3	1.7±0.2
LTB_4, quin2	5.2±0.2	3.8±0.6	1.9±0.1	1.5±0.3
LTB_4, fura-2	5.4±0.4	3.5±0.5	2.0±0.2	2.2±0.3
LTB_4, BAPTA	5.0±0.4	3.8±0.6	1.7±0.2	1.5±0.3

ator loading of these PMN further decreased binding; and exogenously added Ca^{2+} reversed all binding defects (Table 3). Scatchard analyses of binding data for LTB_4 (Fig. 5) revealed that Ca^{2+}-depleted PMN (with or without chelator loading) had 20–50 % fewer high-affinity binding sites than did Ca^{2+}-repleted PMN (Table 4). The K_a for these sites (Table 4) as well as the R_t and K_a for low-affinity binding (results not shown) did not exhibit significant differences among the variably processed cells. Similar changes in high-affinity binding occurred with PAF (Table 4). Again, low-affinity PAF binding was not altered by these treatments (results not shown). With fMLP, in contrast, Ca^{2+}-depletion (with or without chelator loading) produced up to 50 % decreases in the R_t of both high- and low-affinity binding sites (Table 5). High-affinity fMLP binding, however, was exceedingly small, highly variable, and therefore not significantly different in Ca^{2+}-depleted and Ca^{2+}-repleted cells. We conclude that the depletion of cell Ca^{2+} or, alternatively, the lack of extracellular Ca^{2+} (Ca^{2+}-depleted PMN were necessarily suspended in Ca^{2+}-free buffer) decreased the number of receptors available to fMLP, PAF and LTB_4.

Temperature-dependency of the Ca^{2+} effect

For this, 10^7 PMN (with or without chelator loading) were incubated at 37 °C for 20 min in 1 ml of buffer containing 1.4 mM or no Ca^{2+}, then diluted with 8 vol. of 4 °C buffer which contained 0 or 1.4 mM-Ca^{2+} (as in the initial incubation), washed twice and suspended in 4 °C Ca^{2+}-free buffer; then incubated at 4 °C with 1.4 mM or no Ca^{2+} for 10 min, and finally assayed for the specific binding of [^{3}H]agonist. The protocol yielded PMN that had been incubated with 1.4 mM (+) or no (−) Ca^{2+} at 37 °C and then with 1.4 mM (+) or no (−) Ca^{2+} at 4 °C, i.e. +/+, +/−, −/+ and −/− preparations. Results with fMLP and PAF were unequivocal. Only PMN exposed to Ca^{2+} at 37 °C (+/+ and +/−) bound the ligands normally (Table 6). LTB_4 binding to these PMN, perhaps because of the extensive cell-processing procedures, was erratic regardless of the treatment regimen. Accordingly, we used a second protocol. In this, 10^7 PMN (with or without chelator loading) were incubated in 1 ml of buffer containing 1.4 mM or no Ca^{2+} at 37 °C for 20 min, resuspended (2.5×10^6 cells/ml) in 4 °C buffer, incubated with 1.4 mM-Ca^{2+} or 5 mM-EGTA at 4 °C for 10 min, and assayed for the specific binding of [^{3}H]LTB_4. This defined PMN on the basis of their 37 °C exposure to 1.4 mM (+) or no (−) Ca^{2+} and 4 °C exposure

Table 6. Temperature-dependency of the Ca^{2+} effect of agonist binding

PMN were loaded with no chelator, 32 μM-quin2 AM, 32 μM-fura-2 AM or 10 μM-BAPTA in Ca^{2+}-free buffer and washed twice and resuspended (10^7/ml) in this buffer. For fMLP and PAF, PMN were incubated at 37 °C with 1.4 mM (+) or 0 (−) Ca^{2+} for 20 min, transferred to 8 vol. of 4 °C buffer (Ca^{2+} as per initial incubation), washed twice in 4 °C Ca^{2+}-free buffer ([EGTA] = 1 μM), resuspended (5×10^6/ml) in the latter buffer (4 °C), incubated (4 °C) with 1.4 mM (+) or 0 (−) Ca^{2+} for 10 min, and assayed for specific binding. For LTB_4, PMN were processed through the 20 min 37 °C incubation just described, centrifuged, resuspended (5×10^6/ml) in 4 °C buffer, and incubated (4 °C) with 1.4 mM-Ca^{2+} (+) or 5 mM-EGTA (−) for 10 min before assaying LTB_4 binding. Results are the mean percentage of ligand specifically bound, ±S.E.M. for ≥ 10 experiments: * indicates $P > 0.05$ (Student's paired t test) compared with values for PMN loaded with the same chelator and incubated with 1.4 mM-Ca^{2+} during the 37 °C and 4 °C incubations.

	Ca^{2+} presence during the 37 °C and 4 °C incubations			
Ligand, chelator	+/+	+/−	−/+	−/−
fMLP, no chelator	2.0±0.2	1.9±0.2	1.5±0.2*	1.5±0.2*
fMLP, quin2 AM	1.9±0.2	2.3±0.3	1.5±0.2	1.4±0.2*
fMLP, fura-2 AM	1.9±0.3	2.9±0.4	1.4±0.3*	1.3±0.3*
fMLP, BAPTA	2.0±0.3	1.9±0.3	1.4±0.3*	1.4±0.3*
PAF, no chelator	4.6±0.4	4.9±0.4	3.7±0.3*	3.8±0.4*
PAF, quin2 AM	5.6±0.5	5.2±0.6	3.4±0.5*	3.6±0.8*
PAF, fura-2 AM	4.8±0.4	6.0±0.7	3.6±0.6*	3.4±0.7*
PAF, BAPTA	4.9±0.5	4.9±0.4	3.6±0.4*	4.0±0.4*
LTB_4, no chelator	45.1±1.7	43.7±1.7	36.7±3.2*	36.9±1.8*
LTB_4, quin2 AM	43.4±1.5	41.6±1.5	30.3±2.2*	34.8±1.3*
LTB_4, fura-2 AM	43.8±2.9	44.3±1.5	32.8±2.3*	33.8±2.9*
LTB_4, BAPTA	40.5±2.9	41.8±1.4	33.7±1.2*	33.1±2.7*

Table 5. Effects of Ca^{2+} on fMLP-binding parameters in PMN

PMN were prepared as indicated in Table 4. R_t and K_a (±S.E.M.; $n \geq 11$) were determined by the LIGAND program. LIGAND indicated that fMLP bound to high- and low-affinity sites ($P < 0.05$, F-distribution) under all conditions.

	Low affinity		High affinity	
	R_t (receptors/PMN)	$10^{-4} \times K_a$ (M)	R_t (receptors/PMN)	$10^{-9} \times K_a$ (M)
Ca^{2+}-repleted	38000±2000	1.8±0.4	100±40	3.9±0.8
Ca^{2+}-depleted	21000±3000	1.8±0.4	70±40	8.8±0.7
Ca^{2+}-depleted, +quin2	20000±1000	2.0±0.2	70±20	9.2±0.9
Ca^{2+}-repleted, +quin2	43000±600	2.0±0.4	120±60	4.4±0.5

to 1.4 mM-Ca^{2+} (+) or 5 mM-EGTA (−) as +/+, +/−, −/+ and −/−. Again, the 4 °C incubation did not alter results, and only PMN treated with Ca^{2+} at 37 °C (+/+, +/−) showed normal LTB_4 binding (Table 6). Extracellular Ca^{2+} itself thus does not promote agonist binding, and the Ca^{2+} effect appears to be due to receptor changes that occur at 37 °C and persist at 4 °C.

DISCUSSION

Stimulated PMN issue numerous excitatory signals, among which elevated $[Ca^{2+}]_i$ is regarded as essential for the degranulating, O_2^--generating and many other bioactions of standard receptor agonists. PMN loaded with either $\geqslant 25$ μM-quin2 AM [6,7,9,11,12,14,17–21] or > 10 μM of a BAPTA analogue, MAPT AM [15,22], and then suspended in medium containing excess EGTA do not raise $[Ca^{2+}]_i$, release granule enzymes or generate O_2^- when challenged with diverse Ca^{2+}-mobilizing humoral stimuli. We observed the same unresponsiveness in PMN loaded with 32 μM-fura-2 AM, 32 μM-quin2 AM or 10 μM-BAPTA (Figs. 2 and 3, Tables 1 and 2). However, functional losses did not correlate with the blockage of Ca^{2+} transients: PMN depleted of Ca^{2+} and loaded with 0.1–1 μM-fura-2 AM were unable to raise $[Ca^{2+}]_i$ (Fig. 1), yet released appreciable amounts of lysozyme, β-glucuronidase and O_2^- (Fig. 4). Our data therefore disagree with quin2 and MAPT AM studies [6,7,9,11,12,14,15,17–22] which conclude, for example, that the release of granule enzymes and/or O_2^- by PMN require $[Ca^{2+}]_i$ levels to rise above 100 nM for fMLP [12], 200 nM for C5a [18] and 300 nM for LTB_4 [12]. They are much more in accord with the results of Grinstein & Furuya [13], who found that electropermeabilized PMN responded to fMLP even when $[Ca^{2+}]_i$ was fixed at 100 nM by an EGTA-based buffer system. Discrepancies between our findings and other studies on intact PMN probably reflect procedural differences. We incubated PMN with 1 μM-EGTA for 30 min to remove mobilizable Ca^{2+} pools, whereas previous studies used Ca^{2+} ionophores and/or millimolar EGTA to accomplish this. Furthermore, we assayed $[Ca^{2+}]_i$ with fura-2, whereas the cited studies used quin2. Fura-2 has much better intrinsic fluorescence properties, and therefore is used at 10–30-fold lower levels than quin2. The relatively harsh Ca^{2+}-stripping methods and/or high quin2 levels (quin2 and fura-2 have similar Ca^{2+} affinities) may have displaced Ca^{2+} from pools other than those contributing to $[Ca^{2+}]_i$ or, alternatively, produced less specific inhibitory effects. In any case, it seems clear that PMN can mount exocytotic and O_2^--generation responses to the receptor agonists in the absence of appreciable Ca^{2+} transients.

Ambient Ca^{2+} promotes fMLP, PAF and LTB_4 binding in various systems [33–38]. We found the cation to have similar influences on PMN-specific binding of each agonist (Table 3), and moreover to exert its effects temperature-dependently. Thus Ca^{2+}-depeleted PMN (with or without chelator loading) lost agonist-specific binding capacity; this was reversed by incubating PMN with Ca^{2+} at 37 °C, but not at 4 °C; and Ca^{2+}-repleted PMN bound agonists at 4 °C irrespective of ambient Ca^{2+} (Table 6). Since extracellular Ca^{2+} equilibrates with PMN at 37 °C, but not at 4 °C [10,39], our results strongly implicate intracellular Ca^{2+} in agonist binding. Nevertheless, the data of Table 6 cannot exclude a possibility that Ca^{2+} worked outside of the cell. Any such effect, however, would have to occur only at 37 °C and effect changes that persisted at 4 °C independently of extracellular Ca^{2+}: the cation could not act merely by bridging polar areas of ligand/receptor complexes, altering ligand confirmation or state of aggregation, or inducing other changes that require its continuing presence and are so strictly temperature-dependent. Moreover, intracellular Ca^{2+} chelators enhanced the effects of Ca^{2+}-depletion (Table 5). The data suggest, then, that Ca^{2+} acts at some intracellular site to up-regulate agonist receptor numbers (Fig. 5, Tables 4 and 5). This may occur by one of three mechanisms. First, receptors commonly move between internal and surface sites [40]. PMN contain granule-associated fMLP receptors, which shuttle to the plasmalemma [41,42]. Cell Ca^{2+} might promote these receptor dynamics [33] to cause a net accumulation of cell-surface receptors. On the other hand, PMN do not appear to have internal receptors for PAF and LTB_4 [43,44], and the effect of Ca^{2+} on PAF and LTB_4 binding occurs with cell-free membrane preparations [35–39]. Ca^{2+}-induced mobilization of spare receptors does not seem capable of explaining all of our results. Second, bivalent cations promote N-protein/receptor interactions [45–47]. Such interactions convert low-affinity (or non-binding) fMLP, PAF and LTB_4 receptors into higher-affinity (or binding) states [48–50]. Ca^{2+}-depleted PMN may have fewer receptors configured with N-proteins and therefore lower numbers of detectable agonist-binding sites. Third, protein kinase C appears to augment LTB_4-receptor expression in resting PMN [51]. Cellular Ca^{2+} helps to activate this enzyme [1,2], and thereby might exert a tonic effect on receptor expression. This or other Ca^{2+}-sensitive elements could act analogously on PAF and fMLP receptors. Regardless of its exact mechanism, however, we note that Ca^{2+} promotes the expression of the same high- (for PAF, LTB_4), and low- (for fMLP) affinity receptors that are involved in PMN activation [43,44,52]. Hence the decreased responsiveness of Ca^{2+}-depleted PMN may reflect in part these receptor losses. Since LTB_4 and PAF form during PMN stimulation and may then proceed to mediate function, LTB_4- and PAF-receptor losses may act collaterally to decrease PMN responses to other agonists besides LTB_4 and PAF. Nevertheless, losses of receptors for the primary stimulus or secondary mediators probably cannot explain all of the Ca^{2+} influence on function: loading Ca^{2+}-depleted PMN with high levels of fura-2 AM, quin2 AM or BAPTA caused profound losses of responsiveness, but only relatively small decreases in receptor numbers (Fig. 4, Tables 4 and 5). Ca^{2+} therefore seems necessary not only for receptor expression but also for post-receptor events involved in agonist-induced exocytosis and oxidative metabolism.

In conclusion, our data indicate three roles for Ca^{2+} in stimulus transduction. First, Ca^{2+} maintains the full expression of agonist receptors. This Ca^{2+} is readily removed at 37 °C, but not at 4 °C, is influenced by fura-2 AM, and thereby is distinguishable from extracellular Ca^{2+} itself. Second, $[Ca^{2+}]_i$ transients enhance function, perhaps by potentiating the effects of other, more important, response-eliciting signals. The sources of this Ca^{2+} are sensitive to gentle depletion methods as well as to low levels of fura-2. The relationship of this Ca^{2+} pool to that regulating receptor expression is uncertain. Finally, a pool of intracellular Ca^{2+} either triggers or permits function. This pool is relatively insensitive to all but the highest levels of intracellular Ca^{2+} chelators, and it, rather than rises in $[Ca^{2+}]_i$, appears essential for cell responses to fMLP, PAF, LTB_4 and probably other similarly acting receptor agonists.

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